Microbial Keratinases: Characteristics, Biotechnological Applications and Potential

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Abstract

Keratinases are a group of proteolytic enzymes that can catalyse the cleavage and hydrolysis of the highly stable and fibrous proteins: keratins. A diverse range of microorganisms, including fungi, actinomycetes and bacteria, have been reported to produce keratinases that have biotechnological applications and potential. These keratinases have been usefully applied in agricultural, pharmaceutical, leather and textile processes as well as within environmentally friendly waste management solutions. Potential uses of keratinases include the fields of biomedicine, cosmetics, biological control and the generation of green energy. Herein we aim to provide an overview of the properties of this group of versatile enzymes, including the mechanisms of keratin degradation. The diversity of microbial sources of keratinases is discussed and the optimisation of keratianse production examined. We conclude with an assessment of the established biotechnological applications of keratinases in different industries and current research that highlights other promising potential uses.

1 Introduction

Keratinases are key proteolytic enzymes produced by dermaptophytes; they hydrolyse both ‘soft’ (cytoskeletal materials in epithelial tissues, containing up to 1% sulphur) and ‘hard’ (protective tissues in hairs and nails, containing up to 5% sulphur) keratins (Karthikeyan et al. 2007). Hence, in the past few decades, a number of research projects have focused on the activities of keratinases and their role in the virulence of dermatophytes such as *Trichopyton* and *Microsporum* (Siesenop and Bohm 1995; Monod 2008). The potential of

keratinases in the biotechnological context has gained substantial and significant recognition since the beginning of the 21st Century: their substrate specificity and ability to attack highly cross-linked and recalcitrant structural proteins that resist common known proteolytic enzymes, such as trypsin and pepsin, make them valuable biocatalysts in industries that deal with keratinous materials. Novel applications of keratinases are continuously being discovered (see section 6). A number of excellent reviews have been published charting the progress of our understanding of keratinases and their microbial sources, providing excellent overviews on the ecology, physiology and mechanisms of keratinolytic microorganisms (Korniłłowicz and Bohacz 2011) and the applications of keratinases (Onifade et al. 1998; Gupta and Ramnani 2006; Karthikeyan et al. 2007; Brandelli et al. 2010; Guptaet al. 2013a, b). This article aims to consolidate and update the information to provide a comprehensive review of this remarkable biocatalyst.

2 Characteristics and Properties of Keratinases

Keratinases are proteolytic enzymes that can hydrolyse keratins. Keratins belong to a super family of intermediate filaments. They are stable, insoluble and fibrous structural proteins that are found in epithelial tissues (soft epitheria keratins) and protective tissues such as hair, nails and horns (hard trichocytic keratins). Coulombe and Omary (2002) have developed a set of principles for defining the structures, functions and regulations of keratin. The primary function of keratins is to protect cells from mechanical and non-mechanical stress; they also have other roles such as cell signalling, regulating the availability of other abundant cellular proteins and as a stress protein.

In general, keratins can be classified as Type I (acidic keratins) or Type II (basic keratins). The strength and robustness of keratin is derived from the highly stable, tightly packed α-helix (in α-keratins such as hair) and/or β-sheet (in β-keratins such as horn and hooves) configurations. The keratin micro- and macro-filaments in these pleated sheets are supercoiled to form a highly stable left-handed superhelical motif (Voet and Voet 1995) sustained by strong inter- and intramolecular hydrogen bonds and hydrophobic reaction of the polypeptides (Bradbury 1973). In addition, all keratins contain a high degree of cysteine which confers rigidity and chemical resistance via the crosslinking of thermally-stable disulphide bonds. The amount of cystine plays a significant role in determining the nature of the keratin; in soft keratin, the amount of cystine present (up to 2%) was much lower than the hard keratin (~ 22%; Korniłłowicz-Kowalska and Bohacz, 2011). Table 1 lists the cystine content in different types of keratins.

Keratinases are predominantly secreted extracellularly into the growth medium containing keratin (Monod et al. 2002; Gupta and Ramnani 2006; Brandelli et al. 2010). However, Wawrzkiewicz et al. (1987) noted that *Trichophyton gallinae* only produced intracellular keratinase, whilst Korniłłowicz-Kowalska (1999) and Al-Musallam et al. (2013) observed the production of both extracellular and intracellular keratinase in geophilic microscopic fungi namely *Arthroderma quadrifidum*, *A. curreyi* and *Chrysosporium pruinosum*) and macroscopic fungi (*Coprinopsis* sp.) respectively. Gessesse et al. (2003) and Manczinger et al. (2003) reported the production of constitutive keratinase by *Nesterenkonia* sp. AL20 and *Bacillus licheniformis* respectively, whereas Apodaca and Mckerrow (1989) discovered that keratinase may be constitutively produced in *Trichophyton rubrum* in the absence of keratin.

The classification and nomenclature of all proteolytic enzymes are available in the MEROPS database (http://merops.sanger.ac.uk/cgi-bin/family_index?type=P#S). These proteases are grouped into: aspartic, cystein, glutamic, asperagine, metallo, mixed, serine, threonine peptidases and those that are of unknown catalytic mechanisms. Microbial keratinases are predominantly of the metallo, serine or serine-metallo type (Brandelli 2008) with the exception of keratinase from yeast which belongs to aspartic protease (Negi et al. 1984; Lin et al. 1993; Koelsch et al. 2000). Both metallo and serine peptidases are endoproteases that cleave peptide bonds internally within a polypeptide.

Metalloproteases are highly diverse, having more than 90 families. A common feature of this type of enzyme is the involvement of a divalent ion (such as Zn^{2+}) for their catalytic activities which are inhibited by metal chelating agents, transition or heavy metals (Gupta and Ramnani 2006; Riffel et al. 2003; Nam et al. 2002; Thys et al. 2004). Serine proteases fall into two broad categories based on their structure: chymotrypsin-like (trypsin-like) or subtilisin-like. The subtilisin subfamily are completely inhibited by PMSF (phenylmethanesulfonylfluoride), antipain and chymostatin (Tyndall et al. 2005).

2.1 Optimal pH and Temperature

Keratinases belonging to the metallopeptidase group work best in neutral to mildly alkaline conditions (pH 7-8.5; Bach et al. 2011; Sousa et al. 2007; Lee et al. 2002; Tork et al. 2013; Riffel et al. 2007; Han et al. 2012) with the exception of the keratinase produced by *Bacillus thuringiensis* TS2 (Sivakumar et al. 2013) where the optimal pH was 10 and one of the two metalloproteases isolated from an endophytic and keratinolytic *Penicillium* spp. Morsy 1 which also has an optimum working pH range of 10-11 (El-Gendy, 2010).

Keratinases belonging to the serine peptidase group are mainly alkaline proteases that have pH optima in the alkaline range (pH 8-11; Habbeche et al. 2014; Yoshioka et al. 2007 Fakhfakh et al. 2009; Lv et al. 2010; Jeong et al. 2010; Cao et al. 2009). Some alkalophilic actinomyces such as *Nocardiopsis* sp. strain TOA-1 (Mitsuiki et al. 2004) and *Streptomycces* AB1 (Jaouadi et al. 2010); and alkalophilic bacteria *Bacillus circulans* (Benkiar et al., 2013) and *Bacillus halodurans* AH-101 (Takami et al. 1999) have been found to produce keratinases that perform best in a highly alkaline environment (pH >11.5). Atypically, the serine keratinases produced by two fungal strains have an acidic optimal pH range: *Trichophyton mentagrophytes*at pH4.5 (Tsuboi et al. 1989) and *Purpureocillium lilacinum* at pH 6 (Cavello et al. 2013).

Only a few keratinases belonged to the group serine-metalloprotenase have been isolated: *Bacillus* sp. 50-3 (Zhang et al. 2009), *Stenotrophomonas maltophilia* BBE11-1 (Fang et al. 2013), *Streptomyces gulbargensis* (Syed et al. 2009), *Streptomyces* SK1-02 (Letourneau et al. 1998) and *Streptomyces* sp. 7 (Tatineni et al. 2008). This group of keratinases also have alkaline optimum pH range (9-11).

In general, microbial keratinases have a broad, thermally stable range where they can function and the optimal temperature is along the thermophilic range of 45-60 ℃ (Kim 2007; Lateef et al. 2010; Xu et al. 2009; Kojima et al. 2006; Tork et al. 2013; Rai et al. 2009; Sivakumar et al. 2013; Jaouadi et al. 2013; Riffel et al. 2007; Bernal et al. 2006a; Cavello et al. 2012; Sye et al. 2009, Cao et al. 2008; Chao et al. 2007). A number of organisms such as *Actinomadura keratinilytica* Cpt29 (Habbeche et al. 2014), *B. circulans* (Benkiar et al. 2013), *Thermoactinymces candidus* (Ignatova et al. 1999), *Thermoanaerobacter keratinoplilus* (Riessen and Antranikian 2001), *Fervidobacterium pennavorans* (Friedrich and Antranikian 1996; Kluskens et al. 2002) and *F. islandicum* (Gödde et al. 2005) produce keratinases that work best at temperature at or above 70 ℃.The highest optimal temperature (100 ℃) was recorded by Nam et al. (2002) from a serine keratinase, produced by *F. islandicum* AW1, isolated from a geothermal hot spring. Mesophilic keratinses with lower optimal temperature range (20-45 ℃) are predominately produced by pathogenic organisms including *Kocuria rosea* (Bernal et al. 2006a), *Myrothecium verrucaria* (Moreira-Gasparin et al. 2009), *Scopulariopsis brevicaulis* (Malviya et al. 1992), *Serratia marcescens* P3 (Bach et al. 2012), *S. maltophilia* (Fang et al. 2013; Cao et al. 2009; Jeong et al. 2010; Yamamura et al. 2002) and *Trichophyton* sp. (Anbu et al. 2008; Ismail et al. 2012) is which probably indicative of the ecological niches they occupy.

2.2 Biochemical Properties of Keratinases

The majority of keratinases reported are monomeric enzymes with a diverse range of molecular weights (14-240 kDa; see section 3). The keratinase produced by *Bacillus pumilus* A1 has the lowest molecular weight (Fakhfakh et al. 2013), whereas *K. rosea* produced keratinase of the highest molecular weight (Bernal et al 2006a). Although less common, multimeric kerainases have also been isolated in a number of microorganisms. Keratinase from fungal isolates of *Coccidioides immitis* produced seven distinct polypeptides ranging from 15 to 65 kDa (Lopes et al 2008), *S. brevicaulis* and *Penicillium* spp. Morsy 1 both produced two fractions when purified by SDS-PAGE (sodium dodecyl sulphate polyaccylamide gel electrophoresis) ranging from 24-45 kDa and19-40 kDa respectively (Malviya et al. 1992; El-Gendy 2010). Actinomycetous isolates of *Steptomyces* sp. strain 16 produce keratinase comprised of four active polypeptides varying from 19 to 50 kDa (Xie et al. 2010). Multimeric keratinases were also detected in bacterial strains such as *Bacillus* sp. MTS (three fractions ranging from 16-50 kDa; Rahayu et al. 2012); *Chryseobacterium* sp. kr6 (three active fractions at 20-64 kDa; Riffel et al. 2007; Silveira et al. 2010); and *C. indologenes* TKU014 (three active fractions, 40-56 kDa; Wang et al. 2008); *Kytococcus sedentarius* M17C (two fractions at 30 and 50 kDa; Longshaw et al. 2002) and *B. licheniformis* ER-15 (28 and 30 kDa; Tiwary and Gupta 2010).

Keratinase produced by *B. licheniformis* PWD-1 is the best studied and the entire nucleotide sequence of the coding and flanking regions of the keratinase structure gene, kerA, was determined (Lin et al. 1997). Although many microorganisms are able to produce keratinase (see section 3) and many have been sequenced (Gupta et al. 2013b), few keratinase encoding genes have been cloned and expressed in heterologous systems (Radha and Gunasekaran 2007; Porres et al 2002) except for *Bacillus megaterium*, which is a stable host to clone and express keratinase genes from heterologous origin (Radha and Gunasekaran 2007). In contrast, in a comparative study using *Escherichia coli*, *B. subtilis*, and *Pichia pastoris* as cloning hosts to express the keratinase gene from *B. licheniformis* BBE11-1, *B. subtilis* appeared to be the ideal host for keratinase production (Liu et al. 2014).

2.3 Chemical Properties of Keratinases

The N-terminal sequences of a number of keratinases have been comprehensively analysed and reviewed by Gupta and Ramnani (2006) and Brandelli et al. (2010). Depending on the microbial source, keratinases produced by each class and group share a high degree of similarity in their N-terminal sequences (Table 2). Most keratinases isolated from the *Bacillus* sp. belong to the subtilisin group and have very high (>90%) N-terminal sequence homology

with the subtilisin Carlsberg produced by *B. licheniformis*. Keratinases A (kerA) and RP (kerRP) from *B. licheniformis* PDW-1 and RPk respectively are almost identical to subtilisin Carlsberg (Lin et al. 1995; Fakhfakh et al. 2009; Jacobs et al. 1985). The deduced amino acid sequence revealed that the keratinase kerRP differs from kerA, subtilisin Carlsberg, and a keratinase of *B. licheniformis* by 2, 4, and 62 amino acids, respectively but conserving the active site residues D32, H63 and S220 (Fakhfakh et al. 2009). Keratinases from *B. licheniformis* MKU3 and MSK103 have over 99% and 87% similarity with kerA respectively (Radha and Gunasekaran 2007; Yoshioka et al. 2007) and the keratinase from *B. circulans* showed more than 80% homology with *B. pumilus* K12 and *B. pumilus* CBS (Benkiar et al. 2013).The N-terminal amino acid of keratinase KERUS of *Brevibacillus brevis* US575 differs from *B. pumilus* A1, *B. pumilus* CBS and subtilisin Carlsberg by only one amino acid - the Gln13 residue in KERUS was an Ala13 in the other enzymes. Similarly, keratinase isolated from *Streptomyces griseus*, *S. albidolavus K1-02* and *Streptomyces fradiae* share comparable N-terminal sequences, but are distinct from keratinases produced by other bacterial and fungal strains (Table 2).

A number of chemicals have been shown to inhibit keratianses (Table 3). Keratinases belonging to the metalloproteases group are inhibited by metal chelating agents [e.g. ethylenediaminetetraacetic acid (EDTA)], organic ligands (e.g.1,10-phenanthroline)and a number of heavy metals including Cu^{2+},Hg^{2+}, Pb^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+} and Mn^{2+} (Riffel et al. 2007; Thys et al. 2006; Farag and Hassan 2004; Daroit et al. 2011; Sivakumar et al. 2013). Serine proteases are generally inhibited by PMSF (Benkiar et al. 2013; Jaouadi et al. 2013; Xie et al. 2010; Shrinivas et al. 2012) and some are also susceptible to Cd^{2+} and Hg^{2+} inhibition (Anitha and Palanivelu 2013; Benkiar et al. 2013; Chaudhari et al. 2013; Li et al. 2007). Keratinases that are serine-metalloproteases are sensitive to both chelating agents and PMSF (Tork et al. 2013; Tatineni et al. 2008; Fang et al. 2013). The presence of Ca^{2+} and Mg^{2+} appeared to enhance keratinase activities in all protease groups (Farag and Hassan 2004; Benkiar et al. 2013; Sivakumar et al. 2013; Jaouadi et al. 2013; Riffel et al. 2007). Interestingly, whilst Co^{2+} and Cu^{2+} are inhibitory to the metalloproteases produced by *Bacillus* sp. P45 (Dozie et al. 1994), *Bacillus subtilis* NRC 3 (Tork et al. 2013), *B. thuringiensis* (Sivakumar et al. 2013) and some *Chryseobacterium* sp. (Chaudhari et al. 2013; Riffel et al. 2007), they improved the serine protease activities in *B. circulans* DZ100 (Benkiar et al. 2013), *B. brevis* US575 (Jaouadi et al. 2013), *B. licheniformis* BBE11-1 (Liu et al. 2013) and *S. fradiae* var k11 (Li et al. 2007). A small number of keratinases are stimulated by the presence of surfactants and detergents; metalloproteases of *Chryseobacterium gleum* (Chaudhari et al. 2013), serine proteases of *Aspergillus parasiticus* (Anitha and Palanivelu 2013), *Brevibacillus* sp. AS-S10-II (Mukherjee et al. 2011) and *S.*

maltophilia BBE11-1 (Fang et al. 2013) are augmented by Triton X-100, Tween 20, Tween 80 and non-ionic surfactants.

Keratinases that are either active or stable in the presence of organic solvents, surfactants, and bleaching agents have potential industrial applications. A keratinolytic serine protease secreted by *P. lilacinum* is found to demonstrate stable keratinolytic activities with dimethyl sulfoxide (DMSO), methanol, and isopropanol; Triton X-100, SDS, Tween 85 or hydrogen peroxide (Cavello et al. 2012). The keratinase produced by *B. pumilus* KS12 was found to exhibit both high detergent compatibility and oxidation stability with an eight- and five-fold enhancement of enzymatic activities in the presence of Triton X-100 and saponin respectively (Rajput et al. 2010). The keratinolytic proteases of *Meiothermus ruber* H328 was able to tolerate SDS at 30 % (w/v) and organic solvents (methanol, ethanol, acetonitrile, acetone, and chloroform) at 40 % (v/v) at 60 ℃ (Kataoka et al. 2014). Similarly, the thermally stable keratinase isolated from *Meiothermus* sp. I40 also exhibited good stability in the presence of DMSO, ethanol, isopropanol and acetonitrile (Kuo et al. 2012) and the keratinase produced by *B. halodurans* PPKS-2 was not inhibited by SDS, EDTA, H_2O_2 (15%) or other commercial detergents (Prakash et al. 2010a).

It has been reported that reducing agents such as dithiothreitol (DTT), β-mercaptoethanol, cysteine and sodium sulphite stimulated keratinase activity as the thiol groups activate the keratinolytic enzymes (see section 2.5; Gupta and Ramnani 2006; Fang et al 2013; Tatineni et al. 2008; Xie et al. 2010). However, this phenomenon was not universal and did not apply to the keratianses isolated from *Brevibacillus* sp. AS-S10-II (Mukherjee et al. 2011) and *Chryseobacterium* sp. kr6 (Riffel et al. 2007); probably resulting from the chelation of essential ions that are necessary to maintain the structure and activity of the keratinase by DTT (Riffel et al. 2007).

2.4 Keratinous Substrates and their Specificities

Microbial keratinases can be isolated in a number of sources and have diverse properties depending on the producer organisms (Brandelli et al. 2010; see section 3 for details). For example, keratinases from fungi, actinomycetes and bacteria have a wide range of substrates: from soft keratin such as stratum corneum (Błyskal 2009) to hard keratin such as feather keratin (Mazotto et al. 2013; Gousterova et al. 2005; Frie Friedrich and Antranikian, 1996; Ichida et al. 2001), sheep's wool (Farag and Hassan, 2004; Xie et al. 2010; Riessen and Antranikian 2001; Han et al. 2012), human and animal hairs (Chen et al. 2011; Desai et al. 2010; Gurav and Jadhav 2013; Jaoudai et al. 2013), nail, hoof and horn (Mohorčič et al. 2007; Friedrich and Kern 2003; Tiwary and Gupta 2010; Błyskal 2009) and azokeratin (Bach

et al. 2011; Kim 2007). Other substrates that are susceptible to keratinase degradation include: collagen (Fang et al. 2013; Bernal et al. 2006a; Farag and Hassan 2004); elastin (Brandelli et al. 2010; Bressollier et al. 1999); gelatine (Tork et al. 2013; Lopes et al. 2008); albumin and haemoglobin (Benkiar et al. 2013; Lopes et al. 2008), fibrin (Tiwary and Gupta 2010; Tork et al. 2013).

In addition to substrates listed above, keratinase is also able to degrade unusual recalcitrant animal proteins such as prions (Suzuki et al. 2006; Tsiroulnikov et al. 2004; Langeveld et al. 2003). Prions are fatal neurodegenerative transmissible agents causing several incurable illnesses in humans and animals. Prion diseases are caused by the structural conversion of the cellular prion protein, PrP^{C}, into its misfoldedoligomeric form, known as PrP^{Sc} (Abskharon et al. 2014). The normal prion protein PrP^{C} consists of approximately 45% α-helix and only 3% β-sheet, but the abnormal conformer PrP^{SC} consists of approximately 30% α-helix and 45% β-sheet (Pan et al. 1993). This structure shares a high degree of similarity with feather keratin. The feather keratin molecule contains a 32-residue segment that is believed to form the framework of the filament that has a helical structure with four repeating units per turn; each repeating unit consists of a pair of twisted β-sheets related by a perpendicular diad (Fraser and Parry 2007).

The substrate specificity of keratinases is strongly influenced by the chemical properties of their substrates. As keratin is composed of 50-60% hydrophobic and aromatic amino acids (Gradišar et al. 2005; Brandelli et al. 2010), keratinases appear to cleave preferentially hydrophobic and aromatic amino acid residues at the P1 position (Gradišar et al. 2005; Silveira et al. 2009; Brandelli et al. 2010; Gupta et al. 2013a). Hydrolysis studies using oxidised insulin B as the substrate showed that phenylalanine, valine, tyrosine and leucine were selectively cleaved by keratinases of *Thermoanaerobacter* sp. (Kublanov et al. 2009a), *B. pumilus* KS12 (Rajput et al. 2010), *Nesterenkonnia* sp. AL20 (Bakhtiar et al. 2005), *Pseudomonas aeruginosa* KP1 and KP 2 (Sharma and Gupta, 2010a,b) and *Streptomyces* sp. (Tsiroulnikov et al. 2004). Studies using synthetic amino acid *p*-nitroanilide (*p*NA), *p*-nitrophile ester (*O*N*p*) or 7-amino-4-methylcoumarin (AMC) as substrates (Table 3) demonstrated the substrate specificity of these keratinases. The residues in the P2 and P3 positions also play a role in the substrate specificity. N-Succinyl-Ala-Ala-Phe-*p*Na was susceptible to degradation by keratinases of *B. pumilus*, but not its analogue N-Succinyl-Gly-Gly-Phe-*p*Na (Rajput et al. 2010). Macedo et al. (2008) showed that the keratinase KerS14 of *B.subtilus* preferred to cleave Arg at the P1 position, small amino acid residues at the P2 position, and Gln or Glu at the P3 position. Keratianses also seemed to prefer utilisation of longer substrates which may be indicative that the presence of amino acids further along the

cleavage site residue is important to the substrate specificity. This is probably due to the availability of additional active sites (Böckle et al. 1995; Bressollier et al. 1999; Mitsuiki et al. 2004).

2.5 Mechanism of Keratinolysis

Over the years, a number of hypotheses have been proposed to explain the mechanism of keratin degradation by microbial keratinases (Korniłłowicz-Kowalska and Bohacz 2011). Broadly speaking, it is agreed that keratin degradation encompasses two main stages: deamination and keratinolysis (Kunert 1976, 1989; Kaul and Sumbali 1997). Deamination creates an alkaline environment for optimal enzymatic reaction by the alkaline proteases (Kunert, 1989, 1992, 2000; Kaul and Sumbali, 1997). The complex mechanism of keratinolysis that follows involves the cooperative action of sulphitolytic and proteolytic enzymes (Yamamura et al. 2002). Rahayu et al. (2012) noted the degradation activities on natural keratin substrates by purified keratinase from *Bacillus* sp. MTS was enhanced by the purified disulfide reductase, compared to activity of each enzyme alone. This is further supported by the observations of Fang et al. (2013) in which three kertinolytic enzymes (a serine protease, serine-metalloprotease and disulfide reductase) were isolated from *S. maltophilia* BBE11-1and none of these enzymes showed keratinolytic activity independently.

During sulphitolysis, disulphide bonds between polypeptide keratin chains are cleaved and thiol groups liberated. Kunert (1972) showed that, in the presence of sulphite, disulphide bonds of the keratin substrate are directly cleaved to cysteine and S-sulfocysteine. Sulphitolysis changes the conformation of keratin and exposes more active sites, making them accessible for further digestion by alkaline protease and resulting in the release of soluble peptides and amino acids (Yamamura et al. 2002; Kunert 1992; Gradišar et al. 2005; Cao et al. 2008; Böckle et al. 1995; Monod 2008).

It is believed that keratin degradation in keratinolytic fungi also includes an additional mechanical step involving the frond myceliain dermatophytes (Kanbe and Tanaka 1982) and boring hyphae in non-dermatophytes that penetrate the substrate surface (Kanbe et al. 1986). The keratinolytic activities of dermatophytes are higher than the non-dermatophytes (Filipello Marchisio 2000). Amongst non-dermatophytic fungi, soft keratin degraders are likely to produce thin boring hyphae and hard keratin degraders tend to form swollen boring hyphae (Korniłłowicz-Kowalska and Bohacz 2011).

In prokaryotic cells, sulphitolysis can be achieved by the production of disulphide reductases, release of sulphite and thiosulphate (Kunert 1989; Ramnani et al. 2005) or a

cell-bound redox system (Brandelli et al. 2010; Sharma and Gupta 2010a). Disulphide reductases produced by a number of microbes have been shown to effectively reduce the disulphide bonds, specifically: *Streptomyces pactum* (Böckle et al. 1995), *Vibrio* Kr 2 (Sangali and Brandelli 2000), *Stenotrophomonas*D-1 and *S. maltophilia* BBE11-1 sp. (Yamamura et al. 2002; Fang et al. 2013), *Bacillus* sp. MTS (Rahayu et al. 2012) and *B. halodurans* PPKS-2 (Prakash et al. 2010a). It is noted that purified keratinases are generally less effective in hydrolysing native keratin, probably due to the removal of disulphide bond reduction components during the purification process (Nam et al. 2002; Cao et al. 2008; Brandelli et al. 2010). A suitable redox environment may be necessary for effective degradation of keratin. The presence of reducing agents (Böckle et al. 1995; Gradisar et al. 2005; Thys and Brandelli 2006; Cao et al. 2008) or a cell-bound redox system (Ramnani et al. 2005; Ramnani and Gupta 2007; Moreira-Gasparin et al. 2009) stimulate keratin hydrolysis by purified keratinase. In a cell-bound redox system, the bacterial cells probably provide a continuous supply of reductant (e.g. sulphite) to break disulfide bridges (Ramnani et al. 2005; Sharma and Gupta 2010a).

In addition to sulphur-containing amino acids, sulphite is also produced by dermatophytes from environmental cysteine, a process that is governed by the key enzyme cysteine dioxygenase Cdo1, which is then secreted by the sulphite efflux pump Ssu1. As keratin is rich in cysteine, the mechanism of cysteine conversion and sulfite efflux may also play a role in keratin degradation (Grumbt et al. 2013). Kasperova et al. (2013) also suggested that Cdo is a virulence factor, crucial for keratin degradation, as it is involved in the oxidation of cysteine to cysteine sulphinic acid during disulphide bridges cleavage.

3 Sources of Microbial Keratinases

Keratinolytic degraders can be found in diverse groups of microorganisms: from fungi, actinomycetes to bacteria. The origin and substrates of a number of notable keratinase producers are listed in Tables 5-7b. These microorganisms are frequently isolated from keratin-rich environments such as soil and wastewater associated with the poultry industry and tannery wastes.

Dematophytic fungi are amongst the most recognised keratin degraders. Their virulence and pathogenicity have been linked to their ability to degrade both soft and hard keratin (Monod et al. 2002; Brouta et al. 2002; Giudice et al. 2012). However, due to the potential risks of infection, biotechnological applications of these fungi have not been widely explored (Brandelli et al. 2010; Błyskal 2009). A comprehensive review of nearly 300 fungi species

(both pathogenic and non-pathogenic) has been published by Błyskal (2009) detailing their ability to degrade different keratinous substrates. The number of strains that were able to utilise the keratinous substrates were: hair>>wool>feather>textile>hedgehog spine>nail>human plantar callus>hoof>horn (ibid). A number of keratinases produced by non-pathogenic fungi have been isolated and characterised (Table 5); these enzymes showed promising potential applications for a number of industries (see section 5). *Aspergillus* (Kim 2007; Mazotto et al 2013; Anitha and Palnivelu 2013; Farag and Hassan 2004), *Coprinopsis* (Al-Musallam et al. 2013), *Doratomyces* (Friedrich et al. 2005) *Paecilomyces* (Gradišar et al. 2005; Mohorčič et al. 2007; Veselá and Friedrich 2009) *Penicillium* (El-Gendy 2010) and *Purpureocillium* (Cavello et al. 2012) are the most common non-pathogenic fungi that produced keratinolytic activities.

Actinomyces are also known to be a rich source of keratinase (Table 6). A number of mesophilic *Streptomyces* (Böckle et al. 1995; Jaouadi et al. 2010; Bressollier et al. 1999; Gushterova et al. 2005; Szabo et al. 2000; Letourneau et al. 1998; Xie et al. 2010; Tatineni et al. 2008) and thermophic *Streptomyces* sp. (Chitte et al. 1999; Gushterova et al. 2005, 2012; Syed et al. 2009; Ignatova et al. 1999; Vasileva-Tonkova et al. 2009a) produced keratinases that break down keratin. Another promising keratinase was isolated from *Nocardiopsis* sp. TOA-1 and has been demonstrated to degrade synthetic keratin substrate (Mitsuiki et al. 2004), as well as scrapie prion (Mitsuiki et al. 2006).

A number of Gram-positive and Gram-negative bacteria are also found to be important keratinase producers. From the Gram-positive category, members of the *Bacillus* genus are the most prominent and prolific of the keratin degraders (Tables 7a-7b). In particular, keratinases from *B. licheniformis* are capable in degrading feathers (Ichida et al. 2001; Okoroma et al. 2012; Langeveld et al. 2003; Fakhfakh et al. 2009), wool and animal hide (Tiwary and Gupta 2010; Desai et al. 2010) and PrP^{SC} prion (Langeveld et al. 2003; Yoshioka et al. 2007; Okoroma et al. 2013). From the Gram-negative category, keratinases produced by members of the *Chryseobacterium* or *Stenotrophomonas* genera have been widely studied and shown to degrade feather (Chaudhari et al. 2013; Gurav amd Jadhav 2013; Cao et al. 2009; Jeong et al. 2010), animal hair (Gurav and Jadhav 2013; Cao et al 2009), wool (Fang et al. 2013; Cao et al. 2009), hoof and horn (Cao et al. 2009; Yamamura et al. 2002).Some thermophilic anaerobic bacteria also demonstrated an ability to produce serine type keratinases. *Fervidobacterium pennavorans* (Friedrich and Antranikian 1996) and *F. islandicum* (Nam et al. 2002; Kluskens et al. 2002) were isolated from hot springs and produced keratinases that can degrade feathers efficiently. A novel new species of

thermophilic anaerobic bacterium with keratinolytic activities, *Keratinibaculum paraultunense* gen. nov. sp. Nov KD-1, was isolated by Huang et al. (2013) from grassy marshland.

Other less common microbial sources that produce keratinases include several hyperthermophilic archaeons. *Thermoanaerobacter keratinophilus* (Riessen and Antranikian 2001), *Thermoanaerobacter* sp. strains 1004-09 (Kublano et al. 2009a) and VC13 (Tsiroulnikov et al. 2004) are effective in hydrolysing both α and β keratins. In addition, *Thermococcus kodakarensis* produces keratinolytic proteases that degrade PrP^{SC} prion (Hirata et al. 2013; Koga et al. 2014) and *Desulfurococcus kamchatkensis* sp. Nov 1221n^{T} was able to utilise α keratin (Kublanov et al. 2009b). A small number of lichens including *Parmelia sulcata*, *Cladonia rangiferina* and *Lobaria pulmonaria* were also found to produce serine keratinases that could degrade hamster PrP^{TSC} prion (Johnson et al. 2011).

4 Optimisation of Keratinase Production

Production of keratinase from a commercial perspective requires an integrated approach that combines optimal fermentation conditions, operational optimisation and effective downstream processing. Medium composition and culture conditions are the two important factors that affect the yield of an enzyme in a fermentation process. The keratin source usually serves as the sole carbon and nitrogen sources in a growth medium. The addition of separate carbon and nitrogen sources have been shown to increase enzyme production in some microorganisms (Brandelli et al. 2010; Ramnani and Gupta 2004), but suppress production in others (Brandelli and Riffel 2005; Brandelli et al. 2010). It is suggested that as each microorganism has its own optimal set of growth parameters; these conditions should be treated on a case-by-case basis (Cai and Zheng 2009; Brandelli et al. 2010). The most significant parameters that affect keratinase production can be investigated using a one-factor-at-a-time method. Optimisation of the selected components can be achieved using a statistical approach such as employing the Plackett-Burman design and response surface methodology (RSM) to develop a mathematical model to identify the optimum conditions for higher keratinase production (Tiwary and Gupta, 2010; Pillai et al. 2011; Rai and Mukerjee 2011; Haddar et al. 2010; Bernal et al. 2006b; Tatineni et al. 2007; Embaby et al. 2010)). Alternatively, the optimal components concentration can be deduced using a central composite design (CCD), followed by analysis using the RSM (Harde et al. 2011; Daroit et al. 2011; Bach et al. 2012).

Investigations carried out on keratinase production methods have focused predominantly on submerged fermentations (SF; De Azeredo et al. 2006; Brandelli et al. 2010). However, the

use of solid-state fermentation (SSF) has gained prominence as it has a number of advantages over SF, including: lower production expense, smaller water and energy demand, less effluent production and more stable products.Therefore, SSF technology holds a tremendous promise, especially in developing countries(Hölker and Lenz 2005; Mukherjee et al. 2008; Rai et al. 2009). A number of researchers have demonstrated the potential of SSF: De Azeredo et al. (2006) reported higher keratinase activity in *Streptomyces* sp. 594 cultured in SSF than SF. Similarly, keratinolytic activity produced *by Aspergillus niger* strain 3T5B8 using SSF was found to be seven times higher than those recorded in SF (Mazotto et al. 2013). Mukerjee et al. (2008) successfully produced keratinase from *B. subtilis* DM-04 using *Imperata cylindrical* grass and potato peelings (in a ratio of 1:1) as a low-cost medium. Likewise, Rai et al. (2009) obtained β-keratinase from *B. subtilis* strain RM-01 in SSF using a chicken-feather substrate; and Kumar et al. (2010) reported *B. subtilis* MTCC9102 was able to produce a significant amount of keratinase under optimized conditions in SSF using a horn-meal substrate. Da Gioppo et al. (2009) recorded comparable enzymatic activities from keratinase produced by *M. verrucaria* grown in SF and SSF using poultry feather powder and cassava bagasse as substrates. *Paenibacillus woosongensis* TKB2 cultured in SSF conditions using chicken feather as substrate, with rice straw (2:1), moistened with distilled water (1:5, w/v adjusted to pH 8.5) and fermented for 72h, increased the production of a keratinase that can dehair goat hides within 14 h without the addition of lime (Paul et al. 2013a).

The use of immobilised microorganisms as well as purified enzymes has also been investigated. Prakash et al. (2010b) demonstrated that whole-cell immobilization was useful for continuous production of keratinase and feather degradation by *B. halodurans* PPKS-2. A number of materials have been employed to immobilise cell-free keratinase successfully including sintered glass beads, chitin, chitosan beads, biotinylated acrylic beads and nanoparticles. Keratinase of *Aspergillus oryzae* immobilised on sintered glass beads showed a higher thermal stability at 70 °C and longer half-life than the free enzyme (Farag and Hassen 2004). Rajput and Gupta (2013) reported increased enzymatic stability at 70 °C when the keratinase produced by *B. subtilis* immobilised on chitin by covalent crosslinking. Similarly, keratinase of *B. subtilis* immobilised on poly(ethylene glycol)-supported Fe_3O_4 superparamagnetic nanoparticles showed a fourfold increase in the enzymatic activity over the free enzyme; and enhanced thermal stability, storage stability and recyclability were also observed (Konwarh et al. 2009).The thermal stability of the keratinase from *Chryseobacterium* sp. kr6 immobilised on glutaraldehyde-activated chitosan beads also improved around two-fold when compared to the free enzyme at 65 °C, and the immobilised enzyme remained active after several uses (Silveira et al. 2012). *Aspergillus flavus* K-03 also

displayed a higher level of heat stability and an increased tolerance toward alkaline pHs compared with the free keratinase and retained 48% of the original enzyme after 7 days of incubation (Kim 2007). Improved thermal stability and pH tolerance was also observed in a fusion protein of keratinase and streptavidin immobilised on biotinylated acrylic beads, although its rate of reaction were lower than those of the free enzyme (Wang et al. 2003).

5 Established Applications of Keratinases

The ability of microbial keratinases to degrade keratin and other recalcitrant materials holds much biotechnological potential and has generated a significant amount of research interest in the last couple of decades. One of the earliest reviews on the biotechnological applications of keratinases, written by Onifade et al. (1998), documented the potential of these enzymes in producing livestock feeds. Subsequently, other potential biotechnological applications of keratinases have been identified. A number of excellent reviews have extensively examined the use of keratinases in the waste management industry, agroindustry, pharmaceutical and biomedical industries, leather and bioenergy industries (Thanikaivelan et al. 2004; Gupta and Ramnani 2006; Karthikeyan et al. 2007; Brandelli 2008; Brandelli et al. 2010; Korniłowicz-Kowalska and Bohacz 2011; Gupta et al. 2013b). In their review on the biotechnological applications and market potential, Gupta et al. (2013a) provided a detailed survey of keratinases applications, highlighted their uses and provided a list of commercial products involving the use of keratinases.

5.1 Waste Management

A large number of keratinous wastes are generated every year mainly from poultry production and processing, as well as leather and textile industries (Suzuki et al. 2006; Korniłowicz-Kowalska and Bohacz 2011). Approximately 8.5 million metric tonnes of poultry waste was produced worldwide annually; India contributes about 3.5 million tonnes (Gupta et al. 2013a), the United State 1.8 million tonnes and the United Kingdom 1.5 million tonnes (Okoroma et al. 2012). Livestock and poultry farms and slaughter houses also produce a significant number of keratinous wastes in the form of feather, bristles, hair, down, horns and hooves (Braikova et al. 2007; Korniłowicz-Kowalska and Bohacz 2011). Since the outbreak of Bovine Spongiform Encephalopathy (BSE) in the United Kingdom, the European Union and United States have imposed strict guidelines on the use of animal by-products. In the EU, animal by-products are grouped into three categories based on the level of risk in transmitting the pathogens and toxic substances. Only category 3 keratinous wastes can be

processed and used for livestock, pet and fish food, and for composting (Lasekan et al. 2013).

Currently, the poultry industry manages their waste via a number of disposal methods. Carcass and feather wastes are generally rendered into bone, meat and feather meal and then burnt in cement kilns and disposed of in landfill sites (Cascarosa et al. 2012). Diseased mortalities are disposed in disposal pits or incinerated (Nayaka and Vidyasagar 2013). Composting has been championed as an environmentally friendly alternative to manage keratinous wastes (Ichida et al. 2001; Nayaka and Vidyasagar 2013), where organic keratinous wastes are ultimately degraded and converted to inorganic nitrogen (ammonium and nitrate) and sulphurs (sulphates) that can be easily absorbed by plants. Nevertheless, the rate of degradation in compost may be slow due to the recalcitrant nature of keratins and their resistance to normal proteolytic enzymes. Within the compost, the succession is dominated by bacteria and actinomycetes during the first two to four weeks of composting; this is then gradually replaced by fungi. Cellulolytic meso- and thermophilic fungi are the first to emerge while keratinolytic strains are detected in the compost biomass at the sixth week of the process (Korniłłowicz-Kowalska and Bohacz 2010). The growth of keratoinolytic fungi is found to correlate with the mineralisation of organic nitrogen and sulphur in the composted mass (Bohacz and Korniłłowicz-Kowalska, 2009). The addition of keratinase producing microorganisms as an inoculum could, in theory, accelerate and enhance the process. Ichida et al. (2001) showed that by adding *B. licheniformis* and a *Streptomyces* sp. isolated from the plumage of wild birds to compost bioreaction vessels, the bacteria-soaked feathers degraded more quickly and more completely than the controls. Nayaka and Vidyasagar (2013) also demonstrated that the addition of *Streptomyces albus* helped to enhance degradation of chicken feather compost and the release of valuable byproducts acceptable in land use applications. However, Tiquia et al. (2005) failed to observe significant changes in the rate of feather degradation when *B. licheneformis* (OWU 1411T) and *Streptomyces* sp. (OWU 1441) were co-composted with poultry litter and straw; the microbial community structure over time was found to be very similar in inoculated and uninoculated waste feather composts (ibid).

Under laboratory conditions, a number of microbial strains demonstrated their abilities to degrade feathers and other keratins. Chaudhari et al. (2013) observed the dissolution of whole chicken feathers in 72h at 30 °C by *C. gleum*. Thermophilc *B. licheniformis* strain N22 was able to degrade completely melanised feathers in 48 h in the absence of any reducing agent (Okoroma et al. 2012). Complete disintegration of intact feathers into soluble proteins

by keratins was achieved within 7 days at 30 °C by *Serratia* sp. HPC 1383 (Khardenavis et al. 2009) and in 3 days by *Streptomyces* AB1 (Jaouadi et al. 2010).*B. brevis* US575 was able to degrade a range of keratins including whole chicken feathers, rabbit fur and goat hair in 10h at 37 °C. These observations, amongst many others, suggest the potential of using keratinolytic microorganisms in keratinous waste management. The commercial products *Versazyme®* and *Valkerase®* manufactured by BioResource International (BRI) both contain keratinases from *B. licheniformis* and are marketed for recycling of keratin waste (Gupta et al. 2013a)

5.2 Agroindustry

In many ways, keratinous waste management is closely associated to its valorisation; keratinous wastes are rich in protein and can be converted to valuable amino acids by hydrolysis, the resulting hydrolysate is a valuable agricultural resource.

5.2.1 Animal Feed and Feed Supplements

Feather waste contains large amounts of amino acids such as cystine, glycine, arginine and phenylalanine (Onifade et al. 1998), but they have to be hydrolysed to release these valuable amino acids. The processing methods commonly employed to hydrolyse feather waste include thermal, chemical and enzymatic treatments (Papadopoulos 1985). Thermohydrolysis involves heating feather waste at high temperature (80-140 ℃) and pressure (10-15 psi). The treatment is energy intensive, causes the destruction of essential amino acids such as methionine, lysine and tryptophan and creates an additional pollution burden (Papadopoulos 1989; Wang and Parsons 1997). It is thought that the loss in the nutritional value is brought about by the combined effects of the destruction of certain essential amino acids and the reduction in amino acids availability. The latter is caused by the formation of cross-linkages that reduced the rate of protein digestion, possibly by preventing enzyme penetration or by blocking the sites of enzyme attack (Papadopoulos 1989). Physicochemical treatments incorporate organic solvents such as DMSO and dimethyl formamide (DMF), acid or alkali in the keratinous waste to facilitate disulphide bond cleavage, which in turn encourages solubilisation of keratin and the release of amino acids (Coward-Kelly et al. 2006a; Kornilłowicz-Kowalska and Bohacz 2011). However, the amino acid composition of these products are low in arginine, histidine, lysine, methionine and threonine; and, especially for hair waste, the composition compares poorly with the essential amino acid requirements for various monogastric domestic animals (Coward-Kelly et al. 2006b). Digestion experiments carried out on young chicks also showed that sodium hydroxide added during thermal treatment may have a negative effect on the digestibility of

the feed (Papadopoulos 1989). The use of keratinases or keratinolytic microorganisms in the treatment of feather meal overcomes some of the limitations posed by thermal and chemical treatments. Keratinase PWD1 is found to improve the digestibility of keratin and significantly enhance the growth of poultry (Odetallah et al. 2003). The application of *K. rosea* in the production of feather meal has shown to: improve the digestibility of the fermented production; increase the lysine, histidine and methionine content and boost the availability of these amino acids (Bertsch and Coello 2005). The commercial products *Versazyme* produced by BBI and Cibenza DP100™ by Novus International have been marketed as additives to feed to improve their nutritional values (Gupta et al. 2013a).

The nutritional value of animal feeds can also be enriched by the introduction of a hydrolysate supplement, produced by keratinolytic microorganisms (Gupta and Ramnani 2006; Brandelli 2008; Brandelli et al. 2010). Wool protein hydrolysate from *B. pumilus* A1 also presented a very high in vitro digestibility (97%) as compared with that of the untreated wool (3%; Fakhfakh et al. 2013). Similarly, the feather protein hydrolysate of *B. pumilus* A1 presents a signicantly higher digestibility (98%) compared with that of the untreated feathers (2%) as well as possesses antioxidant activity, thus it may be useful as supplementary protein and antioxidants in animal feed formulations (Fakhfakh et al. 2011). The alkaline keratinase produced by *Brevibacillus* sp. strain AS-S10-II converted feather-keratin to at least seven volatile amino acids (cystenine, valine, threonine, lysine, isoleucine, phenylalanine and methionine; Mukherjee et al. 2011). Similarly, feather hydrolysate from *Vibrio* sp. Strain kr2 (Grazziotin et al. 2006) and *Streptomyces* sp. (Ramakrishnan et al. 2011) were found to be effective in improving the nutritional value of feather meals. It has been suggested that since keratin is naturally low in some essential amino acids such as methionine and phenylalanine, the use of keratinolytic microbial cultures may further enrich the hydrolysate by the presence of microbial proteins and biomass (Brandelli et al. 2010; Vasileva-Tonkova et al. 2009a,b; Grazziotin et al. 2006).

5.2.2 Fertilizers

Hydrolysates produced by keratinolytic microorganisms are also ideal as fertilisers or soil amendments due to their high nitrogen and amino acid contents (Brandelli et al. 2010; Vasileva-Tonkova et al. 2009a). Alkaline hydrolyzed sheep's wool (Gousterova et al. 2008) and thermally degraded wool waste (Nustorova et al. 2006) have been shown to be beneficial to both plants and soil microbes as the hydrolyzed product could be readily utilized by the soil microorganisms. Rice seeds treated with feather hydrolysate from *Bacillus* sp. AJ4 and AJ9 demonstrated a 30% increase in vigour index as well as improvement in feed conversion ratio and plant growth (Arasu et al. 2009). Hydrolysates from bovine hooves and

horns using *Paecilomyces marquandii* is also a good source of fertiliser as they contain large quantities of amino acids (except for proline and tryptophan) and compared favourably to other fertilisers in promoting plant growth (Veselá and Friedrich 2009). The filter-sterilised hydrolysate of *P. woosongensis* TKB2, using raw feather as the sole substrate, can promote the germination of seeds and growth of *Cicer arietinu* seedlings significantly; improve nodule formation and increase the soil fertility and can be exploited as a useful biological fertiliser (Paul et al. 2013b).

5.3 Leather and Textile Industry

Leather processing involves three major processes: pre-tanning (beamhouse operation) where hides or skins are cleaned using sodium sulphate (Na_2S) and lime; tanning where the leather materials are stabilised with chromium sulphate ($CrSO_4$), solvent and lime; and post-tanning and finishing where aesthetic value is added.

During the conventional lime-sulphide dehairing process, large amount of Na_2S is involved and the waste generated by this operation causes serious environmental and waste disposal problems. Thanikaivelan et al. (2004) provided a detailed review on a number of biocatalysts that have used in: cleansing and rehydration (soaking); removal of unwanted hair (dehairing); removal of undesirable proteins (bating) and eliminating fat (degreasing). The use of kertinlolytic microorganisms with good dehairing action has been hailed as a promising and viable alternative to chemical dehairing (ibid; Dettmer et al. 2011, 2013). A histological study of porcine skin degradation by *Dormatomyces microsporus* revealed that keratinase first attacked the proteins in the frontiers between the stratum corneum and the rest of epidermis as well as along the border; this is followed by the attack on the epidermal layers beneath the stratum corneum and the outer sheath of hair roots (Friedrich et al. 2005). *B. brevis* US575 has been shown to be effective in removing hair from rabbit, goat, sheep and bovine hides (Rai and Mukerjee, 2011; Jaouadi et al. 2013) and *P. aeruginosa* A2, grown in shrimp shell powder, demonstrated a powerful dehairing capability on bovine hide (Ghorbel-Bellaaj et al. 2012). Enzymatic depilation generally only requires small quantities of Na_2S and could be an eco-friendly alternative to the chemical process. Keratinases from *B. subtilis* S14 (Macedo et al. 2005) and *Trichoderma harzianum* MH-20 (Ismail et al. 2012) could even be applied in the absence of Na_2S. Thus the use of a keratinase-assisted tanning process can significantly reduce the impact of dehairing waste in the environment.

Keratinases produced by a number of *Bacillus* strains (Macedo et al. 2005; Cai et al. 2011; Prakash et al. 2010b; Benkiar et al. 2013), the *Brevibacillus* sp. AS-S10-II strain (Rai and

Mukerhjee 2011), *Microbacterium* sp. kr10 (Thys and Brandeli 2006), *Aspergillus nodulans* (Gupta et al. 2013a), *P. woosongensis* TKB2 (Paul et al. 2013c) and *T. harzianum* MH-20 lack collagenolytic activities. These enzymes are of interest in the bating process as conventional bating enzymes containing collagenase causes physico-chemical changes in the leather (Thanikaivelan et al. 2004). Application of keratinases with low collagenolytic properties can breakdown keratin tissue in the follicle without affecting the tensile strength of the leather (Macedo et al. 2005).

Keratin hydrolysates have also been applied successfully to the tanning and retanning processes. In the conventional chrome tanning process, large amount of unused Cr is discharged into the effluent causing a major pollution concern. The permissible level of Cr in the waste stream is less than 2 mg/L in most countries (Buljan 1996), thus there is a need to improve the Cr uptake in the tanning process. The addition of ketain hydrolysate (2-3% w/w) from horn meal (using *B. subtilis*) helps to reduce the Cr level in the wastewater from 35% to 10% (Karthikeyan et al. 2007). The low molecular weight keratin peptides present in the hydrolysates react with Cr to form a Cr-keratin complex which upon interacting with collagen in the leather enhances the uptake of Cr (Ramamurthy et al. 1989). Keratin hydrolysates are used in the retanning process to improve the properties of leathers; they are used as a filling agent to enhance poor substance skin, grain smoothness and softness (Karthikeyan et al. 2007).

Keratinases also have important applications in the textile industry. A number of microbial keratinases including those from: *B. licheniforms* (Liu et al 2013), *B. cereus* (Sousa et al. 2007), *Chryseobacterium* L99 (Lv et al. 2010) and *Pseudomonas* sp. (Cai et al. 2011) are able to improve felt-shrink resistance and dyeing characteristics with no loss of fibre weight. It is reported that keratinase - acting in combination with other enzymes such as cutinase, lipase and transgultaminase - can be used to further improve the wool processing (Gupta et al. 2013a).

5.4 Consumer Products

A number of consumer products have been known to involve keratinases, from formulation of detergents to personal care products such as shampoo, cosmetics and acne treatment (Brandelli et al. 2010; Gupta et al. 2013b).

5.4.1 Detergent

The application of keratinases in the detergent industry has been most promising as many of these alkaline proteases are thermally stable at wash temperature and tolerant of surfactants

(Rai et al. 2009; Cavello et al. 2012; Prakash et al. 2010a; Rajput et al. 2010). Table 4 presents a number of keratinases that are stimulated by the presence of surfactants and reducing agents which make them ideal candidates for detergent formulation, notably: *A. keratinilytica* Cpt29 (Habbeche et al. 2014), *A. parasiticus* (Anitha and Palanivelu 2013), *Brevibacillus* sp. AS-S10-II (Mukherjee et al. 2011), *C. gleum* (Chaudhari et al. 2013) and *S. maltophilia* BE11-1 (Fang et al. 2013). Due to their substrate specificity, keratinases can clean within a short period of time without damaging the fibre strength and structure (Paul et al. 2014) and a number of keratinases are shown to be capable at hydrolysing keratinous materials that fix on soiled collars and cuffs (Gupta and Ramnani 2006). The alkaline keratinase of *P. woosongensis* TKB2 is effective at removing blood stains from surgical garments and composite stains of blood, egg yolk and chocolate from conventional clothes in a short period without changing the texture of the cloth and cloth fibres (Paul et al. 2014). Similarly, keratinase of *B. thuringiensis* TS2 are also effective in the removal of blood and egg stains as well as depilation of goat hide (Sivakumar et al. 2013). Another application of keratinases in the detergent industry involves their uses in cleaning up drains that are clogged with keratinous waste and keratinous dirt associated with laundry (Brandelli 2008; Farag and Hasan 2004; Itsune et al. 2002). A commercial product, BioGuard Plus, is manufactured by RuShay Inc and marketed for drain pipe and septic tank cleaning (Gupta et al. 2013a).

5.4.2 Personal Care Products

Hair comprises mainly of keratin protein (90%) and a small amount of lipid (1–9%). Keratin hydrolysates are efficient restorers in hair care processes, they contain active peptides that repair and condition the hair (Villa et al. 2013). Most keratin hydrolysates for hair care products are obtained from nails, horns and wool via chemical hydrolysis and hydrothermal methods (Barba et al 2008). However, using microbial keratinases to obtain keratin hydrolysis is also gaining popularity (also see section 5.2). Crude chicken feather hydrolyse produced by *S. maltophilia* is found to be protective to hair, as evidenced by the improved flexibility and strength for both normal and damaged hair (Cao et al. 2012). Villa et al. (2013) successfully formulated a mild shampoo and a rinse off conditioner with the enzymatic hydrolysate which appeared to increase the brightness and softness of hair.

Keratinases also found applications in other personal care products (Gupta et al. 2013a) including: cosmetic skin whitening and bleaching (Yang 2012); exfoliation and removal of stratum corneum (Ding and Sun 2009); removal of corns and calluses (Encarna and Elena 2011); treatment of acne (Spyros 2003) due to the build-up of sebum caused by blockage of hair-shafts by excess keratin; and anti-dandruff shampoo (Selvam and Vishnupriya 2012).

Proteos Biotech produces two types of commercial products: *Keratoclean® Hydra PB* and *Pure100 Keratinase*, for the removal of corns and calluses; and *Keratoclean Sensitive PB* and *Keatopeel PB* for the treatment of acne (Gupta et al. 2013a).

5.5 Pharmaceutical Industry

The two most common diseases affecting the nail unit are onychomycosis (fungal infections of the nail plate and/or nail bed) and psoriasis (an immune-meidated disease causing nail pitting and onycholysis detachment of the nail from the nail bed; Murdan 2002). The nail plate consists mainly of 80% 'hard' keratin and 20% soft keratin (Lynch et al. 1986). In order to deliver an effective topical treatment for nail disease, it is necessary for the hard keratin of nail plate to be weakened or compromised. A number of keratinolytic microorganisms are able to utilise keratin filaments and keratinous tissues as substrates, including: native human foot skin by *Streptomyces* sp. (Xie et al. 2010); native callus and extracted keratin polypeptides by Kytococcus (Longshaw et al. 2002) and human nail plates and clippings by *P. marquandii* (Gradišas et al. 2005; Mohorčič et al. 2007). Using modified Franz diffusion cells and bovine hoof membranes as a model, Selvam and Vishnupriya (2012) demonstrated keratinases increase the permeability, partition co-efficient and the drug reflux of the membrane. In addition, keratinase from *P. marquandii* has been demonstrated to enhance drug delivery by partially hydrolysing the nail plates (Gradišar et al. 2005; Mohoričič et al. 2007). Keratinases are effective instruments to hydrolyse the nail keratins as they cleave the disulphide linkage to increase the access of drug treatment, thus they can act as ungula enhancers (Gupta et al. 2013b). Commercial products involving keratinases for the treatment of nail disorders include FixaFungus™ by FixaFungus and Kernail-Soft PB by Preteos Biotech (Gupta et al 2013a).

The ability of keratinases to hydrolyse keratin can also be applied in wound healing. In third-degree burns, the avascular nature of the wound eschar may prevent effective diffusion of systemic antimicrobial agents to the wound where the amount of microorganisms is usually very high (Manafi et al. 2008). Enzymatic debridement of the wound will enhance penetration of the topically administered antibiotics and encourage wound healing (Krieger et al. 2012). Martínez et al. (2013) developed a gel matrix from enrofloxacin and the keratinase produced by *P. lilacinus* LPS #876, based on a cryogel of PolyVinyl Alcohol – Pectin (PVA – P), for the treatment of wounds and eschars and to regulate the controlled release of antibiotics.

As dermatophytes are prolific keratinase producers, recombinant keratinases have been proposed by a number of researchers as potential candidates for the production of vaccines against dematophytes. A purified recombinant keratinolytic metalloprotease (r-MEP3) was

tested as a subunit vaccine in experimentally infected guinea pigs in order to identify protective immunogens against *Microsporum canis* (Brouta et al. 2003). Although the vaccination induced a strong antibody response, the protocol did not prevent fungal invasion or development of dermatophytic lesions (Vermout et al. 2004). In another investigation, a recombinant keratinase (SUB3) was produced by expressing the virulence factor of *M. canis* in the *Pichia pstoris* expression system. It was found to be non-antigenic to guinea pigs; it elicited specific lymphoproliferative response, but not specific humoral immune response, suggesting SUB3 could be a tool for future vaccination trials in cats (Descamps et al. 2003). Serine proteases produced by *Dermatophilus congolensis* has also been cloned for inclusion in a vaccine to prevent lumpy wool disease (dermatophilosis) using degenerate primers and polymerase chain reaction (Mine and Carnegie, 1997). A novel subtilisin homologue, derived from *Penicillium citrinum*, with IgE antibody binding properties has been identified and demonstrated to have a high degree of homology in the amino acid sequence with the allergen Tri r 2 in *Trichophyton;* this presents the potential of developing a vaccine against *Trichophyton* asthma (Woodfolk 2005).

5.6 Prion Decontamination

PrP^{Sc} has less α-helical content than PrP^{C} and is rich in β-sheet structure (Pan et al. 1993). It is the cause of all neurodegenerative prion diseases (Colby and Prusiner 2011). Infectious prion can be introduced to the environment via a number of routes including: improper disposal of mortalities, shedding of biological materials, effluents from slaughterhouses and hospitals (Bartelt-Hunt and Bartz 2013) and recycling waste products such as bone meal of infected animals (Johnson et al. 2011). Storage and disposal of these clinical and biological wastes is a major public health concern.

Incineration, thermal hydrolysis and alkaline hydrolysis are the common treatments employed to destroy prions. These methods are harsh and energy intensive, they cause irreversible damage to delicate medical instruments and prevent the capture of any recoverable materials (Okoroma et al. 2013). The ability of keratinases to degrade the β-keratin of feathers provides an environmentally friendly and sustainable alternative to degrade prion. Since the earliest report of enzymatic degradation of scrapie prion by Cho (1983), a number of studies have been carried out to explore the applications of microbial keratinases to treat and degrade prion from a number of microbial sources, including proteases from *Bacillus* sp. (Langeveld et al. 2003; Yoshioka et al. 2007; Okoroma et al. 2013), *Streptomyces* sp. (Hui et al. 2004; Tsiroulnikov et al. 2004), *T. kodakarensis* (Hirata et al. 2013), *Nocardiopsis* sp. TOA-1 (Mitsuiki et al. 2006), lichens (Johnson et al. 2011) and other thermophilic organisms such as *Thermoanaerobacter*, *Thermosipho* and

Thermococcus sp. (Suzuki et al. 2006). The keratinase produced by *B. licheniformis* PWD-1 is able to degrade brain stem tissue from cattle infected with bovine spongiform encephalopathy (BSE) and sheep infected with scrapie in the presence of detergent and at elevated temperature (>100 °C; Langeveld et al. 2003). *B. licheniformis* N22 can produce a keratinase that degrades scrapie prion to undetectable levels in the presence of a biosurfactant using Western Blot and cell culture assay within 10 min at 65 °C (Okoroma et al. 2013). Similarly, keratinases from *Thermoanaerobacter* subsp. S290 and *Streptomyces* subsp. S6 have been shown to degrade brain homogenates of mice infected with the 6PB1 BSE strain (Tsiroulnikov et al. 2004). The keratinase E77 from *Streptomyces* sp. (Hui et al. 2004) and NAPase from *Nocardiopsis* (Mitsuiki et al. 2006) can degrade hamster brain homogenate containing scrapie prions. The enzymes extracted from *P. sulcata*, *C. rangiferina* and *L. pulmonaria* are able to reduce prion protein from transmissible spongiform encephalopathies (TSEs) infected hamsters, mice and deer (Johnson et al. 2011).

Three commercial keratinase-based enzymes are marketed for degradation of infectious prion proteins: Versazyme® is manufactured by BRI, Pure100 Keratinase™ is produced by Proteos Biotech and Prionzyme™ produced by Genencor International (Gupta et al. 2013a). Coll et al. (2007) measured the effectiveness of Versazyme® in degrading BSE prion in meat and bone meal (MBM). They found that the enzyme catalysed the hydrolysis of MBM to improve the solubility of insoluble proteins, and it was more effective against bone than soft tissue particles. Prionzyme™ is currently the only effective enzyme-based decontamination technology that demonstrates significant removal of prion from medical and dental instruments (Gupta et al. 2013a).

Composting may also serve as a practical and economical means of disposing of specified risk materials or animal mortalities potentially infected with prion diseases. A thermophilic condition and alkaline environment is highly conducive for microbial keratinase activity (see section 5.15.1). A number of studies have demonstrated biodegradation of prion protein using compost (Huang et al. 2007; Xu et al. 2013). In a field trial, Xu et al. (2014) also observed that composting reduced PrP^{TSE}, resulting in one 50% infectious dose (ID50) remaining in every 5,600 kg of final compost for land application.

6 Potential Applications of Keratinases

In addition to the established biotechnological applications, there are a number of potential applications that utilise the ability and stability of keratinase to hydrolyse keratin over a range of temperature and pH and in the presence of alkaline or reducing agents.

6.1 Biological Control

The potential for keratinases to act as a biological control agent has been explored by several research groups recently. Keratinase produced by *S. maltophilia* R13 is effective against several fungal pathogens including *Fusarium solani*, *F. oxysprum*, *Mucor* sp. and *A. nigar* that cause diseases in valuable plants and crops (Jeong et al. 2010). Similarly, keratinase produced by *Thermoactinomyces* also showed antifungal properties against these plant pathogens (Gousterova et al. 2012). Yue et al (2011) reported that the keratinase produced by *Bacillus* sp. 50-3 has the ability to work effectively against agricultural pests such as toot-knot nematodes (*Meloidogyne incognita)*.

In insects, the tracheae are found on the exoskeleton and each tracheal tube is lined with a thin strip of cuticle called the *taenidia* which reinforces the tracheae to maintain the structure of the tracheal walls. As insect tracheal taenidia contains a protein similar to the vertebrate keratins (Baccetti et al. 1984), this protein may present a possible target for keratinase hydrolysis to control harmful insects such as mosquitoes that are the major vectors of a number of serious tropical diseases. The use of two recombinant baculoviruses containing the *ScathL* gene from *Sarcophaga cylindric* (vSynScathL) and the keratinase gene from *Aspergillus fumigatus* (vSynKerat) has been successful in destroying the larvae of an agricultural pest, *Spodoptera frugiperda*, by degrading extracellular matrix proteins and interfering with the phenoloxidase activity of the insect host (Gramkow et al. 2010). Tangentially, keratinase hydrolysate can be used as a substrate for pesticide production. Poopathi and Abidha (2007) found that poultry waste is a low-cost and effective substrate to cultivate *Bacillus sphaericus* and *B. thuringiensis* serovar *israelensis* to produce mosquitocidal toxin.

6.2 Green Energy

Conversion of keratinous waste into biofuel is a promising application to generate green energy that may address some of the global demand for energy. In a two-step formation process, keratinous waste was first hydrolysed by *B. lichenifomis* and the hydrolysate was subsequently utilised by *Thermococcus litoralis* to produce biohydrogen gas (Bálint et al. 2005). In a comparison study, bacteria from *Thermoanaerobacterales* are found to be more efficient in substrate conversion than *Clostridiaceae* and *Enterobacteriaceae* (Rittmann and Herwig 2012). Production of methane can also be achieved by combining the biological degradation of keratin-rich waste with keratinase in an anaerobic digester. Chicken feather waste pre-treated with a recombinant *B. megaterium* strain showing keratinase activity prior

to biogas production, was able to produce methane in the order of 0.35 Nm^3/kg dry feathers, corresponding to 80% of the theoretical value on proteins (Forgács et al. 2011, 2013).

6.3 Silk Degumming

Keratinases also hold potential for degumming silk. Natural raw silk is composed primarily of fibroin (62.5–67%) and sericin (22–25%; Mahmoodi et al. 2010). Sericin is a fibrous protein that binds the fibroin fibres together; it renders the raw silk harsh and stiff and reduces the effectiveness of dye uptake by the material. During the degumming process, sericin is hydroblised and solubilised in degumming agents and media (Chopra and Gulrajani 1994). A number of proteases have examined for their ability to degum silk (Arami et al. 2007), but some appeared to be only suitable for treating Murshidabad silk (Chopra and Gulrajani 1994.) and many appeared to be low in specificity towards sericin (Freddi et al. 2003). The use of proteases combined with ultrasonic treatment is found to improve the effectiveness of the degumming process and improve the properties of silk yarn such as strength and elongation (Mahmoodi et al. 2010). The application of a more substrate specific enzyme, such as keratinase from *B. subtilus* (Cai et al. 2008) that does not hydrolyse silk, may further improvement of the process.

6.4 Other Applications

As keratinase is a specific type of alkaline protease, it may find applications in areas that are currently the domain of other alkaline proteases. For example, alkaline proteases of *B. pumilus* and *Staphylococcus auricularis* are able to inhibit biofilm formation by 86% and 50% respectively as well as recover 0.4013 g and 0.3823 g of silver from 1 g of X-ray and photographic films respectively (Bholay et al. 2012). Alkaline proteases from *Aspergillus versicolor* (Choudhary 2013) and *B. subtilis* ATCC 6633 (Nakiboglu et al. 2001) also provide good Ag recovery from X-ray films. Other novel and emerging applications of keratinases include removal of cerumen (earwax), pearl bleaching and processing of edible bird's nests (Gupta et al 2013a).

7 Conclusion

Keratinases are versatile and valuable enzymes that degrade keratins and similar recalcitrant proteins. Increased awareness of their biotechnological applications and potential has provided strong impetus to study this group of alkaline proteases. Diverse groups of microorganisms are able to produce keratinases and more are being discovered every year. Knowledge of their chemical and biochemical characteristics improves the

understanding that is needed to fully explore their value. Of the more established biotechnological applications, keratinases have proven to be highly effective in management and valorisation of keratinous wastes and nutritional improvement of animal feed. Keratinase hydrolysates offer eco-friendly alternatives to improve the dehairing, tanning and retanning processes and reduce damage to the environment caused by the chemical discharge of the leather industry. Within the laundry and pharmaceutical industries, keratinases are used in improved detergent formulations, prion decontamination, enhanced drug delivery and personal care products such as nail and acne treatments. Other biotechnological prospects for keratinases are continuously being explored and investigated. The use of keratinases as biological control agents is an exciting prospect for the agroindustry and the public health domain. The involvement of keratinases and their hydrolysates in bioenergy production may help to alleviate some of the global energy demand from unsustainable sources. Microbial keratinases also promise to improve silk degumming and recovery of valuable resources such as silver from X-ray films. Novel applications of keratinases continue to emerge as research advances. Further understanding of the molecular characteristics, enzyme kinetics and the use of recombinant technology may help to broaden the substrate specificity and the applications of this important group of enzymes.

Table 1.Cystine content of keratin

Types of keratin	***Cystine content***	***Example***	***Reference***
Soft keratin	Up to 2%	Epithelial cells – low chemical resistance and mechanical strength	Korniisłowicz-Kowalska and Bohacz 2011
Hard keratin			
α (40-68 kDa)	10-17%	Wool and hair	Robbins 2012 Filipello Marchisio 2000
β (10-22 kDa)	5-10%	Scales and claws -	Dalla Valle et al 2010
γ (amporphic keratin)	~22%	Outer layer of hair cuticle; globular, about 15 kDa, high in sulphur content and acts as disulphide crosslinkers	Robbins 2012; Hill et al 2010
Feather (contain both α-helix and β-sheet*	8%	Feather	Akhatar and Edwards 1997

*feather mainly consists of feather-specific β-keratins, cellular and biochemical studies have shown that α-keratin plays an important role in the early formation of rachides, barbs, and barbules (Alibardi and Toni 2008)

Table 2. N-terminal amino acid sequences of a number of keratinases and their microbial sources.

Microorganism	*Keratinase*	*N-terminal sequence*	*Reference*
Bacteria			
Bacillus circulans DZ100	Keratinase SAPDZ	AQTVPYGMAQIKDPAVHGQGYKGAN	Benkiar et al 2013
Bacillus licheniformis	Subtilisin Carlsberg	AQTVPYGIPLIKADK	Jacobs et al. 1985
Bacillus licheniformis PWD-1	Keratinase A	AQTVPYGIPLIKADK	Lin et al. 1995
Bacillus licheniformis RPk	Keratinase RP	AQTVPYGIPLIKADK	Fakhfakh et al. 2009
Bacillus licheniformis MP1	Alkaline protease	AQTVPYGIPLIKAD	Jellouli et al. 2011
Bacillus mojavensis A21	Serine proteases BM1	AQSVPYGISQIKA	Haddar et al. 2009
	Serine proteases BM2	AIPDQAATTLL	
Bacillus pumilus	Keratinase A1	AQTVPYGIPQI	Fakhfakh-Zouari et al. 2010a,b
B. pumilus	Keratinase CBS	AQTVPYGIPQIKAPAVHAQGY	Jaouadi et al. 2008
Bacillus subtilis	Keratinase S14	AQSVPYGISQIKAPA	Macedo et al. 2005
Bacillus subtilis	Subtilisin E	AQSVPYGISQIKAPA	Stahl and Ferrari 1984
Bacillus subtilus	Keratinase KS-1	AZPVEWGISZ	Suh and Lee 2001
Bacillus halodurans	Keratinase AH-101	SQTVPWGISFISTQQ	Takami et al. 1999
Bacillus pseudofirmus	Keratinase FA30-01	XQTVPXGIPYIYSDD	Kojima et al. 2006
Brevibacillus brevis US575	Keratinase KERUS	AQTVPYGIPQIKEPAVHAQGYKGANVK	Jaouadi et al. 2013
Pseudomonas aeruginosa	Keratinase Pa	AEAGGPGG	Lin et al. 2009
Fervidobacterium pennivorans	Fervidolysin	STARDYGEELSN	Kluskens et al. 2002
Vibrio metschnikovii J1	Serine protease	AQQTPYGIRMVQADQLSDVY	Jellouli et al. 2009
Actinomyces			
Streptomyces griseus	Protease B (SGPB)	ISGGDAIYSSTGRCS	Jurasek et al. 1974
Streptomyces fradiae	Keratinase Sfase-2	IAGGEAIYAAGGGRC	Kitadokoro et al. 1994
Streptomyces albidoflavus	Serine protease SAKase	XXGGDAIYSSXXRXS	Bressollier et al. 1999
Norcardiopsis TOA-1	NAPase	ADIIGGLAXYTMGGX	Mitsuiki et al. 2004
Fungi			
Paecilomyces marquandii	Keratinase Pm	ALTQQPGAPWGLG	Gradisăr et al. 2005
Doratomyces microsporus	Keratinase Dm	ATVTQNNAPWGLG	Gradisăr et al. 2005
Aspergillus fumigatus	Kertainase Af	ALTTQKGAPWGLGSI	Noronha et al. 2002

Table 3. Substrate specificity of some keratinases using synthetic substrate

Microbial source	***Substrate***	***References***
Bacillus licheniformis	↓ N-sccinyl-Ala-Ala-Pro-Phe-*p*NA	Rozs et al. 2001
Bacillus licheniformis PWD-1		Evans et al. 2000
Bacillus pumilus KS12		Rajput et al 2010
Bacillus pumilus A1		Fakhfakh-Zouari et al. 2010b
Paecilomyces marquandii and *Doratomyces microsporus*		Gradišar et al. 2005
Pseudomonas aeruginosa		Lin et al. 2009
*Streptomyces fradiae*var k11		Li et al. 2007
Thermoanaerobacter sp.		Kublanov et al. 2009a
Trichophyton vanbreuseghemii		Moallaei et al. 2006
*Bacillus pumilus*KS12	↓ N-sccinyl-Ala-Ala-Pro-Leu-*p*NA	Rajput et al 2010
Bacillus licheniformis	↓	Rozs et al. 2001
Bacillus pumilus KS12	N-sccinyl-Ala-Ala-Ala—*p*NA	Rajput et al 2010
Lysobacter sp. AL10	↓	Allpress et al. 2002
Microbacterium sp. kr10	CBz-Phe-*p*Na	Thys and Brandelli 2006
Bacillus licheniformis	↓ Bz-Phe-Val-Arg-*p*Na	Rozs et al. 2001
Baciilus subtilis	↓ Bz-Ile-Glu-Gly-Arg-*p*Na	Macedo et al. 2008
Streptomyces pactum DSM 40530	↓ CBz-Phe-*o*Np	Böckle et al. 1995
Nesterenkonia sp. AL20	↓ N-Suscinyl-Leu-Leu-Val-Tyr-AMC	Bakhtiar et al. 2005
Chryseobacterium sp.	↓ L-Leu-AMC	Silveira et al. 2009

↓ - cleavage; P1 position

CBz – Carboxylbenzoyl group; Bz – Benzoyl group; AMC — 7-Amido-4-methylcoumarin

Table 4. Some chemical compounds that attenuate keratinase activities

Microbial source	***Protease type***	***Inhibitors***	***Stimulators***	***Referecnes***
Actinomadura keratinilytica Cpt29	Serine	PMSF, DFP, Ni^{2+}, Cd^{2+}, Hg^{2+}, Ba^{2}+, Fe^{2+}	H_2O_2, Tween 20, Tween 80, Triton X-100, Ca^{2+}, Mn^{2+}	Habbeche et al. 2014
Aspergillus parasiticus	serine	PMSF; Cd^{2+}, $Cu^{2+}$and Zn^{2+}	Ca^{2+}, Mg^{2+} and Mn^{2+} , non-ionic detergents and urea	Anitha and Palanivelu 2013
Aspergills oryzae	metallo	EDTA, Pb^{2+}; Cd^{2+} and Hg^{2+}	Ca^{2+}, Ba^{2+}, Cu^{2+}, Na^{+}, K^{+}, Mg^{2+}	Farag and Hassan 2004
Bacillus sp. P45	metallo	EDTA, SDS, Zn^{2+}, Cu^{2+}, Co^{2+}	Ca^{2+}, Mg^{2+}	Daroit et al. 2011
Bacillus circulans DZ100	serine	PMSF, DFP, Ni^{2+}, Cd^{2+}, Hg^{2+}	Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}	Benkiar et al. 2013
Bacillus halodurans JB 99	serine	PMSF,		Shrinivas et al. 2012
Bacillus licheniformis BBE11-1	serine	PMSF	Mg^{2+}, Co^{2+}	Liu et al. 2013
Bacillus pumilus	serine	PMSF		Kumar et al. 2008
Bacillus subtilis NRC 3	serine-metallo	PMSF, EDTA, citric acid, 1-10-PA, Zn^{2+}, Cu^{2+}, Co^{2+}, Mn^{2+}	Na^{+}, K^{+}, Mg^{2+}	Tork et al. 2013
Bacillus thuringiensis	metallo	EDTA; Cu^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}	Ca^{2+}, Mg^{2+}	Sivakumar et al. 2013
Brevibacillus brevis US575	serine	PMSF, DFP, Cd^{2+}, Hg^{2+}, Ni^{2+}	Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}	Jaouadi et al. 2013
Brevibacillus sp. AS-S10-II	Serine	PMSF, IAA, DTT	SDS, Triton X-100, Tween-20, H_2O_2	Mukherjee et al. 2011
Chryseobacterium gleum	metallo	EDTA, Cu^{2+}, Hg^{2+}	Triton X-100, Tween 80, MCE, Fe^{2+}, Fe^{3+}	Chaudhari et al. 2013
Chryseobacterium sp. kr6	metallo	EDTA, EGTA, PHEN, MCE, DTT, SDS, Cu^{2+}, Zn^{2+}	Ca^{2+}, Mg^{2+}, Cd^{2+}	Riffel et al. 2007
Lysobacter NCIMB 9497	metallo	EDTA		Allpress et al. 2002
Microbacterium sp. strain kr10	metallo	EDTA, PHEN, CMB, Cu^{2+}, Hg^{2+}, Zn^{2+}, Mn^{2+}		Thys et al. 2006
Streptomyces fradiae var k11	serine	PMSF; Co^{2+} and Cr^{3+}	Ni^{2+} and Cu^{2+}	Li et al. 2007
Streptomyces sp. 16	4 x serine	PMSF;	EDAC, DTT, Na^{2+}	Xie et al. 2010
Streptomyces sp. S7	serine-metallo	PMSF, EDTA, SDS	DTT	Tatineni et al. 2008
Stenotrophomonas maltophilia BE11-1	K1: serine-metallo	K1: EDTA, PMSF, SDS, Fe^{3+}	K1: Na^{2+}; Tween 20	Fang et al. 2013
	K2: serine	K2: PMSF; SDS; Fe^{3+}	K2: Ca^{2+}, Na^{+}, DTT, Tween 20	
	K3: disulphide reductase	K3: Fe^{3+}, Cu^{2+}; Mn^{2+}, Zn^{2+}	K3: EDTA, Na^{2+}, DTT, Triton X-100, Tween 20, DMSO	

p-chloromercuribenzoic acid (CMB); diisopropyl fluorophosphates (DFP); dimethyl sulphoxide (DMSO); iodoacetate (IAA); dithiothreitol (DTT); 1- Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC)ethylenediaminetetraacetic acid (EDTA); ethylene glycol tetraacetic acid (EGTA); β-mercaptoethanol (MCE); 1,10-phenanthroline (PHEN); phenylmethanesulfonyl fluoride (PMSF); sodium dodecyl sulphare (SDS)

Table 5. Notable fungal keratinase producers, their origins, properties of the keratinases and their substrates

Microorganism	***Origin***	***Protease Type***	***Molecular weight (kDa)***	***Optimal pH (range)***	***Optimal temperature (range) ℃***	***Substrate***	***References***
Aspergillus flavus K-03	soil	serine	31	8 (7-10)	45 (30-70)	azokeratin, azocasein	Kim 2007
Aspergillus niger	mutated laboratory strains: 3T5B8, 9D40, 9D80, and 11D40	3T5B8 and 9D80: serine 9D40 and 11D40: metallo-	All strains: 60 and 9D80 also produced a band at 130	5	-	casein, bovine serum albumin (BSA), ovalbumin, feather meal, feather keratin, human hair, sheep's wool	Mazotto et al. 2013
Aspergillus parasiticus	soil	serine	36	7	50	keratin	Anitha and Palanivelu 2013
Aspergillus oryzae	marine sediment	metallo	60	Immobilised: 7.0-7.4 Free enzyme: 8	immobilised: 60 free enzyme: 50	BSA and casein keratin, chicken feathers, collagen, duck feathers, sheep wool	Farag and Hassan 2004
Candida parapsilosis	feather waste	serine	60	-	-	native feather	Vermelho et al. 2010
Doratomyces microspores MZKI B-399	type strain	serine	30	8-9	50	porcine skin skin keratins, nail keratins, hair keratins	Friedrich et al. 2005 Friedrich and Kern 2003
Paecilomyces marquandii MZKI B-639	type strain:	serine	33	8 (6-11)	60-65	human nail plates and clippings	Gradišar et al. 2005; Mohorčič et al. 2007
Penicillium spp*. Morsy 1*	marine soft coral *Dendronephthya hemprichii*	metallo	I:19 II:40	I:7-8 II:10-11	I:50 II:60-65	poultry waste	El-Gendy, 2010
*Purpureocillium lilacinum (*formerly *Paecilomyces lilacinus)*	soil	serine	37	6 (4-9)	60 (20-65)	hair waste, blood	Cavello et al. 2012, 2013
Scopulariopsis brevicaulis	-	serine	KI: 40-45 KII: 24-29	KI:40 KII: 35	7.8	human hair	Malviya et al. 1992
Scopulariopsis brevicaulis	marine sponge	-	28	7-7.5 (4-11)	50 (30-80)	soluble keratin from chicken feathers	Sankar et al. 2014
Trichoderma atroviride F6	decayed feather	serine	21	8-9 (4-11)	50-60 (26–70)	casein, gelatin, BSA, feather, synthetic substrates	Cao et al. 2008
Trichopyton sp. HA-2	soil	serine	34	8	35	chicken feathers	Anbu et al. 2008

Table 6. Notable actinomyces keratinase producers, their origins, properties of the keratinases and their substrates

Microorganism	***Origin***	***Protease Type***	***Molecular weight (kDa)***	***Optimal pH (range)***	***Optimal temperature (range) ℃***	***Substrate***	***References***
Actinomadura keratinilytica strain Cpt29	poultry compost	serine	29.2	10 (3-10)	70	keratin keratin azure	Habbeche et al. 2014
Nocardiopsis sp. strain TOA-1	tile-joint	-	19.1	12.5	60	synthetic substrate	Mitsuiki et al. 2004, 2006
Steptomyces pactum DSM 40530	type strain	serine	30	7-10	40-75	keratin azure, feather meal and chicken feather	Böckle et al. 1995
Streptomyces AB1	soil	serine	30	11.5	75	keratin azure	Jaouadi et al. 2010
Streptomyces albidoflavus	soil (hen house)	serine	18	40-70	6-9.5	fibrous keratin, collagen, soluble keratin, gelatin, elastin, orcein	Bressollier et al. 1999
Streptomyces gulbargensis	soil	serine- metallo	46	8 (7-9)	45 (30-60)	feather meal	Syed et al. 2009
Streptomyces S.K$_{1-02}$	naturally degraded feather	serine -metallo				casein, keratin	Letourneau et al. 1998
Streptomyces sp.	soil	4 x serines	I: 25 II:50 III:34 IV:19	(7.5-10)	III: 50 (40-55) IV:60-84	keratin azure, human hair, cock feather, and collagen	Chao et al. 2007; Xie et al. 2010
Streptomyces sp.7	soil from slaughter house	serine -metallo	44	11	45	keratin azure	Tatineni et al. 2008
Thermoactinomyces candidus	degrading sheep wool	serine	30	8.6	70	native keratins	Ignatova et al. 1999

Table 7a. Notable Gram-positive bacterial keratinase producers, their origins, properties of the keratinases and their substrates

Microorganism	***Origin***	***Protease Type***	***Molecular weight (kDa)***	***Optimal pH (range)***	***Optimal temperature (range) ℃***	***Substrate***	***References***
Bacillus sp.	soil from slaughter house and poultry farm	-	32	8	37	azokeratin	Deivasigamani et al. 2008
Bacillus sp. 50-3	faeces of *Agamid* lizard *Calotes versicolor*	serine-metallo	-	10	60	azokeratin	Zhang et al. 2009
Bacillus sp. P7	fish intestine	Serine	-	9 (8-12)	55	feather keratin	Corrêa et al. 2010
Bacillus sp. SCB-3	soybean waste	metallo	134	7	40	-	Lee et al. 2002
Bacillus cereus	wool samples	metallo	45.6	7	45	azocasein, azocoll, keratin azure and wool	Sousa et al. 2007
Bacillus circulans	slaughter house wastewater	serine	32	12,.5	85	keratin, casein, albumin, haemoglobin	Benkiar et al. 2013
Bacillus halodurans PPKS-2	rice mill effluents	serine, disulphide reductase	30 66	11 (7-13)	60-70	hair from goat hide	Prakash et al. 2010a
Bacillus licheniformis ER-15	soil	serine	dimeric 58 (30+28)	11 (7-12)	70 (30-80)	feather, haemoglobin, hooves, fibrin and meat protein, buffalo hide	Tiwary and Gupta, 2010
Bacillus licheniformis KI8102	soil from poultry farm	-	32	7.5	50	human hair, bovine hair, wool	Desai et al. 2010
Bacillus licheniformis MSK103		serine	26	9-10	60-70	prion-infected bovine brain homogenate	Yoshioka et al. 2007
Bacillus licheniformis N22	primary effluent and poultry waste	-	28	8.5 (7-10)	50	melanised feather	Okoroma et al. 2012, 2013
Bacillus licheniformis PWD-1	type strain		33	7.5	50	feather keratin, azokeratin	Lin et al. 1992 Langeveld et al. 2003
Bacillus pseudofirmus FA30-01			27.5	8.8-10.3 (5.1-11.5)	60 (30-80)	azokeratin	Kojima et al. 2006
Bacillus pumilus	cow hide	serine	65	8 (7.5-10)	35 (25-45)	bovine hair	Kumar et al. 2008
Bacillus pumilus A1	slaughter house wastewater		14	10	45	feather	Fakhfakh-Zouari et al. 2010a,b; Fakhfakh et al. 2013
Bacillu pumilus SK12	soil	serine	45	10	60	azo-casein, casein, gelatin, haemoglobin, elastin, feather keratin, fibrin, keratin azure, and α-keratin	Rajput et al. 2010
Bacillus subtilis	poultry waste	3 x serines	54-100	9	50	human hair; Feathers	Mazotto et al. 2010; Villa et al. 2013

Bacillus subtilis NRC-3	compost waste, soil	metallo	32	7.5-8 (5-10)	40-50 (20-60)	gelatine, casein, haemogobin, albumin, collagen and fibrin	Tork et al. 2013
Bacillus subtilis RM-01	soil	serine	20.1	8 (5-8)	50 (25-55)	chicken feather	Rai et al. 2009
Bacillus subtilis SLC	soil	serine	-	10 (2-12)	60	keratin, gelatin, casein, and haemoglobin	Cedrola et al. 2012
B. subtilis 1271, B. licheniformis 1269 and B. cereus 1268	agroindustrial residues from a poultry farm	serine	*B. subtilis* and *B. licheniformis*: peptidases and keratinases in the 15-140 kDa range *B. cereus*: keratinase: 200	10	40-50	chicken feather	Mazotto et al. 2011
Bacillus thuringiensis	soil from feather dumping site	metallo-	-	10 (4-11)	50 (30-80)	azokeratin	Sivakumar et al. 2013
Brevibacillus	Soil	-	83.2	12.5	45	goat skin	Rai and Mukherjee 2011
Brevibacillus sp. Strain AS-S10-II	mutated strain	serine	55	9-10 (5-11)	37 (25-55)	chicken feather	Mukherjee et al. 2011
Brevibacillus brevis US575	soil	serine	29.1	8 (5-11)	40 (20-55)	feather meal, chicken feather, rabit hair, goat hair, bovine hair	Jaouadi et al. 2013
Clostridium sporogenes bv. Pennavorans bv. Nov.	muds near the Solfatara volcano	-	28.7	8	55	collagen, elastin and feather keratin	Ionata et al. 2008
Keratinibaculum paraultunense gen. nov. sp. Nov KD-1 (anaerobic)	grassy marchland	serine		8.0-8.5 (6.0-10.5)	70	-	Huang et al. 2013
Kocuria rosea	soil	serine	240	10 (8-11)	40 (10-60)	keratin, collagen, gelatine and casein	Bernal et al. 2006a; Bertsch and Coello, 2005
Kytococcus sedentarius M17C (formerly Micrococcus)	type strain	serine	P1:30 P2:50	P1:7.1 P2:7.5	P1:40 P2:50	natural, insoluble human callus	Longshaw et al. 2002
Meiothermus sp. 140	hot spring	serine	76	8	70	chicken feather, dove feather, duck feather, human hair, wool, and hog bristle	Kuo et al. 2012
Microbacterium sp. kr10	decomposed chicken feather	metallo	42	7.5	50	feather, casein, gelatin, keratin, BSA and haemoglobin	Thys and Brandelli 2006
Nesternkonia sp. AL20	soil	Serine	23	10 (4-11)	70 (40-80)	casein	Gessesse et al. 2003
Thermoanaerobacter keratinophilus (anaerobic)	soil	serine	135	Intracellular: 7 Extracellular: 8	Intracellular:60 Extracellular: 85	native keratin	Riessen and Antranikian, 2001
Thermoanaerobacter sp. Strain 1004-09 (anaerobic)	hot spring	Serine	150	9.3	60	albumin, gelatin, casein, α and β keratin	Kublano et al. 2009

Table 7b. Notable Gram-negative bacterial keratinase producers, their origins, properties of the keratinases and their substrates

Microorganism	***Origin***	***Protease Type***	***Molecular weight (kDa)***	***Optimal pH (range)***	***Optimal temperature (range) ℃***	***Substrate***	***References***
Chryseobacterium gleum	type strain	metallo	36	8	30	keather	Chaudhari et al. 2013
Chryseobacterium indologenes TKU014	soil	3 x metallo	40-56	5-11	30–50 ℃	keratin	Wang et al. 2008
Chryseobacterium L99 sp. nov.	-	serine	33	8	40	keratin azure	Lv et al. 2010
Chryseobacterium sp. kr6	feather	metallo	64, 38, 20	8.5	50-60	keratin azure	Riffel et al. 2007; Silveira et al. 2010, 2012; Brandelli 2005
Chryseobacterium sp. RBT	soil from poultry waste site			8.6	50	chicken feathers goat's hair	Gurav and Jadhav 2013
Fervidobacterium pennavorans	hot spring	serine	130	10	80	feathers	Friedrich and Antranikian, 1996
Fervidobacterium islandicum AW1	geothermal hot spring	serine	>200 (97 subunits)	9	100	soluble keratin; casein	Nam et al. 2002
Fervidobacterium islandicum DSMZ 5733	hot spring	serine	76	8 (6-8.5)	80 (60-80)	feather	Kluskens et al. 2002
Lysobacter A03, Arthrobacter A08 *and Chryseobacterium A17U psychrotolerant*	Penguin feather	A03: serine A08 and A17U: metallo	-	7-8.5	15-20	feather	Pereira et al. 2014
Lysobacter NCIMB 9497	type strain	metallo	148		50	keratin azure	Allpress et al. 2002
Paracocuss sp WJ-98	soil	metallo	50	6.8 (6-8)		-	Lee et al. 2004
Psuedomonas aeruginosa	marine water	metallo	34	8	60	shrimp waste bovine skin	Ghorbel-Bellaaj et al 2012
Psuedomonas aeruginosa C11	soil	metallo	33	7.5 (5-10)	60	feather, collagen, gelatin, casin	Han et al. 2012
Psuedomonas aeruginosa SK1	soil	serine	45	9	60	feather, fibrin, inoculum and meat protein	Sharma and Gupta 2010a
Serratia sp. HPC 1383	tannery sludge	serine	-	10	60	feather	Khardenavis et al. 2009
Stenotrophomonas maltophilia BBE11-1	poultry farm soil	K1:serine-metallo; K2:serine; K3:sisulphide reductase	K1: 48 K2: 36 K3: 17	K1 & K2: 9 (7-11) K3: 8	K1: 40-60 K2 & K3: 40	casein, BSA, feather, wool, collagen	Fang et al. 2013
Stenotrophomonas maltophilia L1	decomposed poultry	serine	35.2	7.8	40	feather, hair, wool, horn	Cao et al. 2009
Stenotrophomonas sp.	deer fur	serine and disulphide reductase	40 and 15	8 and 8	30	casein, human hair, bovine hoof, collagen	Yamamura et al. 2002
Xanthomonas maltophilia strain POA-1	-	serine	36	9	60	keratin	De Toni et al. 2002

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